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Quantitative analysis of the effect of zidovudine, efavirenz, and ritonavir on insulin aggregation by multivariate curve resolution alternating least squares of infrared spectra☆

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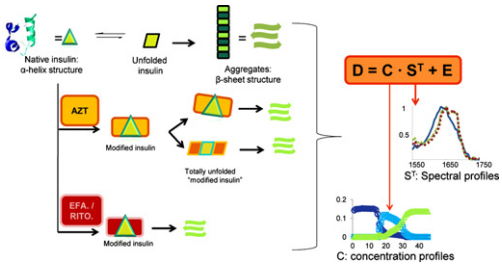
HIGHLIGHTS

- The structure of insulin can be changed via interaction with antiretroviral drugs.
- The chemical interaction promotes the formation of aggregates.
- This drug effect was evaluated by MCR-ALS coupled to IR spectroscopy.
- Formation of aggregates was favourable if drugs were able to form hydrogen bonds.
- Higher drug concentrations favoured formation of amorphous aggregates.

GRAPHICAL ABSTRACT



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ABSTRACT

Quantification of the effect of antiretroviral drugs on the insulin aggregation process is an important area of research due to the serious metabolic diseases observed in AIDS patients after prolonged treatment with these drugs. In this work, multivariate curve resolution alternating least squares (MCR-ALS) was applied to infrared monitoring of the insulin aggregation process in the presence of three antiretroviral drugs to quantify their effect. To evidence concentration dependence in this process, mixtures at two different insulin:drug molar ratios were used. The interaction between insulin and each drug was analysed by ^{1}H NMR spectroscopy. In all cases, the aggregation process was monitored during 45 min by infrared spectroscopy. The aggregates were further characterised by scanning electron microscopy (SEM). MCR-ALS provided the spectral and concentration profiles of the different insulin–drug conformations that are involved in the process. Their feasible band boundaries were calculated using the MCR-BANDS methodology. The kinetic profiles describe the aggregation pathway and the spectral profiles characterise the conformations involved. The retrieved results show that each of the three drugs modifies insulin conformation in a different way, promoting the formation of aggregates. Ritonavir shows the strongest promotion of aggregation, followed by efavirenz and zidovudine. In the studied concentration range, concentration dependence was only observed for zidovudine, with shorter aggregation time obtained as the amount of zidovudine increased. This factor also affected the aggregation pathway.

1. Introduction

Insulin is a small protein consisting of two polypeptides, an A chain (21 amino acids) and a B chain (30 amino acids), linked by two interchain disulphide bridges [1,2]. Insulin adopts a largely α-helical structure in its native state and rapidly undergoes the aggregation process, losing its native structure [3–5]. As

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a consequence, the biological activity of insulin is inactivated and becomes harmful to the organism's, leading to disorders including amyloidosis and prion-associated encephalopathies [6,7]. Controlling the aggregation process is an important challenge in the pharmaceutical field, because it affects the stability of insulin when used as a pharmaceutical product [8,9].

There is extensive literature on the insulin aggregation problem, but the effect of antiretroviral drugs on this process is scarcely documented [10]. Prolonged treatment of AIDS patients with antiretroviral drugs results in metabolic abnormalities such as insulin resistance. Insulin resistance is a disorder in which higher insulin concentrations are required to exert a normal biological response [11,12]. Recently, we have qualitatively reported that insulin remains stable in its native state for less time in the presence of zidovudine, but no information about the quantitative effect of this drug on the aggregation process has been presented [13].

To reduce the adverse effects of antiretroviral therapy, it is important to quantify the effects of different drugs and to also to understand the underlying mechanism of these effects. The mechanism of insulin aggregation is still under review but is widely accepted to begin with unfolding of insulin, followed by its association (nucleation), to finally obtain the aggregates rich in β-sheet structures [14–16].

There are different types of antiretroviral drugs classified according to the phase of the retrovirus life-cycle that they inhibit. The most common types are: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and integrase inhibitors [17]. The first three groups are the most widely studied, and zidovudine (NRTI), efavirenz (NNRTI) and ritonavir (PI) were selected here.

The aim of the present work was to quantify the effect of antiretroviral drugs on insulin aggregation, by applying multivariate curve resolution alternating least squares (MCR-ALS) to the infrared spectra recorded during the process. Infrared spectroscopy was chosen to monitor the aggregation process as it is highly sensitive e to structural changes of proteins and to the specificity of the amide I region for detecting chemical situations related to the loss of native proteins structure [18–20]. Analyses were carried out at two different insulin:drug molar ratios, to determine the concentration dependence for each drug in this process. MCR-ALS is a well-known methodology that has often been used to understand complicated biological systems, as demonstrated by a series of recent publications [21–27].

The present study was conducted in several stages. First, the interaction between insulin and each drug was proved by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Comparison of the individual spectrum of insulin with the spectra of the mixtures it formed with each drug showed that new peaks emerged in the amide region in all cases. This indicates that insulin interacts with each drug to form new peptide/amide bonds. The formation of aggregates was characterised by scanning electron microscopy (SEM) in all cases, and a different morphology to that of native insulin aggregates was observed. Second, the effect of each drug was quantitatively assessed by multivariate-curve resolution alternating least squares (MCR-ALS) of the infrared spectra recorded over time. The number of significant species involved in the aggregation process was determined by inspecting the size of the singular value, calculated by means of singular value decomposition (SVD). The kinetic concentration profiles retrieved by MCR-ALS indicate that all three drugs promote aggregation, but that different aggregation pathways exist depending on the drug type and relative concentration. To evaluate the rotational ambiguity of the MCR-ALS solutions recovered in the individual analysis of each experiment, the feasible band boundaries associated with each of the obtained solutions were calculated using the MCR-bands method [28].

2. Methodology

2.1. Chemical reagents

The drug 3′-azido-3′-deoxythymidine (zidovudine) was purchased in a commercial chemist (GlaxoSmithKline, London). Efavirenz and ritonavir were purchased from Toronto Research Chemicals. CD_3OD was the solvent used for ^{1}H NMR analysis, purchased from SDS. Zn-free human insulin in a HEPES sodium salt buffer (pH = 8.2) at a concentration of 10 mg mL^{-1} (1.72 mM) was purchased from Sigma–Aldrich and used without further purification. All reagents were used as received. The chemical structure of each drug and a schematic representation of insulin are shown in the supplementary material (Fig. S-1).

2.2. General procedure

2.2.1. ^{1}H NMR spectroscopy

Each antiretroviral drug was mixed at two concentration levels with insulin, to obtain insulin:drug mixtures with a molar ratios of 1:1 and 1:2. The ^{1}H NMR spectra of native insulin and each drug were also acquired for comparative analysis. Immediately, 500 μL of each reaction mixture was transferred to a 5 mm NMR tube, using a double tube system. The external reference tube (o.d. 2 mm, supported by a Teflon adapter) containing the reference substance (sodium-3-trimethylsilyl[2,2,3,3-d4]propionate (TSP) 9.9 mmol L^{-1}, $MnSO_4$ 0.47 mmol L^{-1} in 99.9% D_2O) was placed coaxially in the NMR sample tube (o.d. 5 mm). This double tube system was kept at 4 °C in the sample changer until being analysed. ^{1}H NMR spectra were recorded at 300 K on a BrukerAvance III 600 spectrometer operating at a proton frequency of 600.20 MHz using a 5 mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) gradient cryoprobe. One-dimensional ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY)-presaturation sequence (RD-90°–t_1–90°–t_m–90° ACQ) to suppress the residual water peak. t_1 time was set at 4 ms and tm (mixing time) at 100 ms. The 90° pulse length was calibrated for each sample and varied from 7.18 μs to 7.31 μs. The spectral width was 20 ppm, and a total of 64 transients were collected into 64 k data points for each ^{1}H spectrum.

2.2.2. FTIR-ATR

The same experimental procedure was used to prepare the mixtures (insulin–drug) for infrared monitoring. In this case, to take the measurements a drop of the mixture was placed on a small diamond crystal in the spectrophotometer ATR cell (FTIR 680 Plus JASCO and a RS232 Control), which was continuously purged with N_2. The FTIR spectra were recorded in situ every minute, for 45 min, in the spectral range 1550–1750 cm^{-1}. The CO_2 contribution was removed with the control software Spectra Manager before the spectra were exported to Matlab [29]. All experiments were carried out at 37 °C to mimic human body conditions. A solution of human insulin was also monitored using the same experimental conditions to provide comparative analysis. In addition, the infrared spectrum of each drug dissolved in the insulin buffer (HEPES) was acquired. The infrared spectrum of pure zidovudine was recorded during 45 min to evaluate the noise.

2.3. Multivariate curve resolution-alternating least squares (MCR-ALS)

The 45 infrared spectra from each experiment were arranged as rows in D matrices of dimensions 45 × 209. First, Savitzky–Golay smoothing was applied to all data sets to suppress instrumental noise. Assuming a bilinear model, the matrix D can be decomposed